Cellular and Molecular Targets of Estrogen in Normal Human Breast Tissue

Pankaj Seth, Dale Porter, Jaana Lahti-Domenici, Yan Geng, Andrea Richardson, and Kornelia Polyak

Abstract
To gain insight into the in vivo role of estrogen, we isolated estrogen receptor-positive cells from normal human breast tissue using a recombinant adenovirus that expresses green fluorescence protein in response to estrogen. We compared the global gene expression profile of these estrogen receptor-positive cells with that of various normal and cancerous mammary epithelial cells and identified several genes not implicated previously in estrogen signaling. One of these genes, lipocalin 2, is a putative in vivo estrogen target gene and paracrine factor that mediates the growth regulatory effects of estrogen in normal breast epithelium. These results demonstrate that normal and cancerous estrogen receptor-positive cells are distinct at the molecular level and suggest that lipocalin 2 is a new therapeutic target for breast cancer prevention and treatment.

Introduction
Estrogen exposure is one of the most well-recognized risk factors for breast cancer, yet there is relatively little known about the identity of the cells that respond to estrogen in normal breast tissue. The action of estrogen is mediated by its receptors (ERα and ERβ), which act as ligand-dependent transcription factors (1). On the basis of immunohistochemical studies 5–10% of luminal mammary epithelial cells express ER,3 and contrary to ER+ breast cancer cells these normal ER+ cells do not proliferate in response to estrogen (2, 3). In contrast, cells surrounding normal ER+ cells are proliferating frequently, indicating that paracrine factors may mediate the mitogenic effects of estrogen in the normal mammary epithelium (3). To characterize the response to estrogen in normal human breast tissue at the cellular and molecular level, we isolated ER+ cells using a recombinant adenovirus and analyzed their gene expression profiles using SAGE.

Materials and Methods
Generation of Recombinant Adenoviruses. To generate an estrogen-responsive GFP-expressing adenovirus, we gentamizered the 5ERE cassette containing five EREs from the rat PR promoter, fused it to the distal promoter for breast cancer, yet there is relatively little known about the identity of the cells that respond to estrogen in normal breast tissue. The action of estrogen is mediated by its receptors (ERα and ERβ), which act as ligand-dependent transcription factors (1). On the basis of immunohistochemical studies 5–10% of luminal mammary epithelial cells express ER,3 and contrary to ER+ breast cancer cells these normal ER+ cells do not proliferate in response to estrogen (2, 3). In contrast, cells surrounding normal ER+ cells are proliferating frequently, indicating that paracrine factors may mediate the mitogenic effects of estrogen in the normal mammary epithelium (3). To characterize the response to estrogen in normal human breast tissue at the cellular and molecular level, we isolated ER+ cells using a recombinant adenovirus and analyzed their gene expression profiles using SAGE.

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3 The abbreviations used are: ER, estrogen receptor; SAGE, serial analysis of gene expression; GFP, green fluorescence protein; ERE, estrogen-responsive element; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; PR, progesterone receptor; NER, normal estrogen receptor.

epithelial cell line was a generous gift of Dr. Shay (University of Texas Southwestern Medical Center, Dallas, TX). Cells were grown in medium recommended by supplier. To assay estrogen responsiveness, breast cancer cells were cultured in phenol red-free RPMI 1640 or DMEM/F12 medium (Life Technologies, Inc. Rockville, MD) supplemented with 5% charcoal dextran-treated fetal bovine serum (Hyclone, Logan, UT) for 7 days after which cells were switched to fresh medium, or fresh medium containing 10 nM estradiol or 10 μM 4-hydroxytamoxifen. Cells were collected 16–24 h after hormonal treatment.

Fluorescence Microscopy and FACS Analysis. For fluorescence microscopy analysis breast cancer cell lines were infected with Ad-25ERE-GFP virus at a multiplicity of infection ~100 and treated with hormones as described above. Images of cells were obtained 48 h after infection and hormone treatment using a Nikon microscope and a SPOT CCD camera (Diagnostics Instruments, Sterling Heights, MI). For FACS analysis cells were collected by trypsinization, resuspended in ice-cold PBS, and analyzed on an Epics flow cytometer (Beckman Coulter, Fullerton, CA). For the generation of SAGE libraries ~100,000 GFP-positive cells were sorted into medium followed by centrifugation and freezing on dry ice.

Western Blot Analysis. For immunoblot analysis cell extracts were resolved by PAGE, transferred to Immobilon membranes, and blotted with antihuman ER (Ab-11, clone1D5; Neomarkers, Fremont, CA) or antihuman tubulin (Ab-3, clone DM1B; Neomarkers) antibodies.

Generation and Analysis of SAGE Libraries. Normal human mammary epithelium was collected from 18–24-year-old healthy women undergoing reduction mammoplasty at the Brigham and Women’s Hospital using protocols approved by the Institutional Review Board. Organoids were isolated as described previously (5) except that cells were trypsinized and resuspended in phenol red-free DMEM/F12 medium (Life Technologies, Inc.) supplemented with MEGM SingleQuots (Clonetics, Walkersville, MD) and 20 nM of estradiol. Immediately after plating, cells were infected with Ad-25ERE-GFP adenovirus at a multiplicity of infection of ~100. Later (48 h), cells were trypsinized and GFP-positive cells (~100,000 cells) were sorted out by FACS followed by mRNA preparation using μMACS kit (Miltenyi Biotec, Auburn, CA) or Western blot analysis. SAGE libraries were generated and analyzed as described previously (5, 6). NER+–specific genes listed in Table 1 were selected based on pair-wise comparison and Monte Carlo analysis between the NER+ library and each of the SAGE libraries listed in Table 1.

RNA Isolation, RT-PCR, and Northern Blot Analysis. RNA isolation, RT-PCR, and Northern blot analyses were performed as described (5).

mRNA in Situ Hybridization and Immunohistochemical Analysis. mRNA in situ hybridization and immunohistochemical analysis of adjacent sections using anti-ERα antibody (clone 1D5; Dako, Carpinteria, CA) was also performed as described (6).

Generation of Lipocalin 2 Mammalian Expression Constructs and Colony Assays. For constitutive expression the human lipocalin 2 cDNA with a COOH-terminal HA tag was subcloned into pCEP4 (Invitrogen, Carlsbad, CA). For colony assay experiments cells were transfected with pCEP4 (control) or pCEP4-lipocalin-HA constructs using FuGene6 (Roche, Indianapolis, IN) followed by selection in hygromycin containing medium for 2 weeks after which colonies were visualized by crystal violet staining. Expression of lipocalin 2 was confirmed by Western blot analysis of cells and medium using anti-HA antibody (Covance, Richmond, CA). Conditioned medium was generated by infecting COS7 cells with recombinant adenoviruses expressing GFP or lipocalin 2–HA. Filtered medium collected 3–4 days after infection was applied to MCF10A cells. Colonies were visualized by crystal violet staining after 7 days.

Generation of Lipocalin 2 Promoter Reporter Constructs and Luciferase Assays. Lipocalin 2 promoter reporter constructs were generated by subcloning a PCR generated fragment containing the proximal lipocalin 2

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promoter region with (−916 to +50) or without (−800 to +50) the putative ERE into pBPl-huc (7). Cells were transfected using FuGene6 (Roche), treated with 10 nM estrogen, 1 μM p53/ BGT A kinase, and luciferase and β-galactosidase activities were determined 48 h after transfection using a luciferase assay system (Promega, Madison, WI) and the诺vitro assay. The transfection was determined using the Wilcoxon rank sum test. The significance of the differences between the estrogen responsiveness of the two constructs was determined using the Wilcoxon rank sum test. The untreated and estrogen-treated, and the ICI and tamoxifen-treated samples were combined into one group for each construct to achieve the sample numbers necessary for the analysis.

Results and Discussion

Generation and Characterization of the Ad-25ERE-GFP. To isolate ER+ cells from normal human breast tissue, we developed a recombinant adenosovirus (Ad-25ERE-GFP) that expresses the GFP gene only in the presence of ER and estrogen (Fig. 1a). The ER and estrogen dependency of Ad-25ERE-GFP was confirmed by infecting various estrogen receptor-positive and -negative human breast cell lines in the presence or absence of estrogen or tamoxifen (Fig. 1, B and C; data not shown). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno- blot analysis of cell extracts from unsorted and GFP-positive sorted cells (Fig. 1G). To demonstrate that these GFP-positive cells express ER, we performed immuno-
Gene Expression Profile of Estrogen-responsive Cells Isolated from Normal Breast Tissue. To characterize these NER+ cells at the molecular level we generated SAGE libraries from estrogen treated Ad-25ERE-GFP-infected and GFP-positive cells using a modified micro-SAGE protocol (5, 6). We obtained 34,632 SAGE tags from the NER+ SAGE library enabling us to analyze the expression levels of close to 14,000 unique transcripts. Because the SAGE tag numbers directly reflect the abundance of the mRNAs, SAGE data obtained from different experiments are directly comparable. Therefore, to identify genes only or most abundantly expressed in NER+ cells, we compared the NER+ SAGE library to several other SAGE libraries generated by us or available from public sources (5, 7, 8). These included SAGE libraries generated from normal luminal mammary epithelial cells (N1 and N2), ductal carcinoma in situ (D1 and D2), invasive breast carcinomas (I1 and I2), lymph node metastases (M1 and M2), and ER+ breast cancer cell lines (ZR75–1 and MCF-7) in the absence (ZU and MU) or presence of estrogen (ZE, ME3, and ME10) or tamoxifen (ZT). Among these samples, normal luminal epithelial cells, and tumors D1, I1, and M1 were exclusively or mostly ER−, whereas tumors D2, I2, M2, and the two cell lines were ER+. On the basis of pair-wise comparisons and statistical analysis we identified 35 transcripts that were only or most abundantly present in NER+ cells (Table 1). Genes expressed in NER+ cells were of diverse cellular function, and almost none of them corresponded to estrogen target genes characterized previously. However, several of these genes have been shown to be highly expressed in estrogen target organs including the mammary gland, uterus, and ovaries. Claudin-4, kallikrein 5, S100A2, and HE4 were found to be up-regulated in ovarian carcinomas when compared with corresponding normal tissue (9, 10). Similarly the expression of keratin 5/6 and 16, S100A2, and lipocalin 2 was found to be different in normal mammary epithelium, and benign and malignant breast tumors (11–15). Two of the genes, GABA A receptor subunit and lipocalin 2 have been implicated in steroid hormone signaling. GABA A receptor is particularly abundant in the uterus, its protein level fluctuates during pregnancy, and it may be involved in binding endogenous steroids such as pregnenolone (16, 17). The expression of lipocalin 2 (oncogene lipocalin 2 expression and promoter analysis have revealed) has been demonstrated to fluctuate during the estrous cycle in mice with the highest expression correlating with the estradiol surge in proestrus (18). Moreover, the uterine expression of lipocalin 2 was completely dependent on ovarian steroids, because it disappeared in ovariec-tomized mice (18).

Normal and Cancerous Estrogen-responsive Cells Show Distinct Gene Expression Patterns. To additionally investigate the genes highly abundant in NER+ cells, we analyzed their expression levels by Northern blot analysis using RNA prepared from multiple organoids and breast cancer cell lines (Fig. 2A; data not shown). Organoids are breast ducts composed of luminal and myoepithelial cells with a fraction of luminal epithelial cells being ER+. Correlating with the SAGE data many of the genes we identified were highly expressed in normal mammary organoids but not in ER+ breast cancer cell lines (Fig. 2A). In contrast, 5 of 12 ER-negative breast...
cancer cell lines expressed high levels of lipocalin 2 (data not shown) suggesting that its lack of expression in ER+ breast cancer cell lines is unlikely to be only because of differences between normal and cancer cells. We also determined the expression of genes that SAGE predicted to be expressed in ER+ breast cancers but not in NER+ cells, or expressed in both cell types. These former genes included trefoil factors 1 (pS2) and 3, intestinal cysteine rich protein 1, and fatty acid synthase, whereas the later ones included heat-shock proteins 10 and 27, and Mat-8 phospholemma-like fatty acid synthase, whereas the later ones included heat-shock proteins 10 and 27, and Mat-8 phospholemma-like protein (Fig. 2A). These Northern blot results confirmed our previous findings that ER+ cells from normal and cancerous mammary epithelium each express a unique set of genes that could explain their differing response to estrogen.

To investigate additionally the link between estrogen signaling and the expression of some of the genes highly abundant in NER+ cells, we analyzed the expression pattern of their orthologues in mammary glands of virgin, ovariectomized-un-treated and estrogen-treated, pregnant, and lactating mice (Fig. 2, B and C). The expression of lipocalin 2 and, to a lesser degree, S100A2 appeared to be dependent on the presence of estrogen (Fig. 2B). In addition, the mRNA levels of lipocalin 2 were highly induced in lactating mouse mammary gland and gradually increased during pregnancy, but because of the multiple hormonal changes that occur during pregnancy it is not possible to attribute the changes seen to estrogen only (Fig. 2, B and C). Thus, some of these genes could be valid in vivo targets of ER in the normal mammary gland.

**Lipocalin 2 Is a New Candidate in Vivo Estrogen Target Gene.** To demonstrate that at least some of the genes we isolated are expressed in ER+ cells in vivo we performed mRNA in situ hybridization studies. We detected strong hybridization signal in a fraction of normal luminal epithelial cells using antisense lipocalin 2 and S100A2 probes, whereas hybridization with sense probes gave no or a much fainter background signal (Fig. 2D). Immunohistochemical analysis of adjacent sections using anti-ER antibodies suggested that both lipocalin 2 and S100A2, and ER are expressed in an overlapping subset of luminal mammary epithelial cells (Fig. 2D). Lipocalin 2 has been demonstrated to up-regulate its own expression through an autocrine mechanism that could lead to elevated lipocalin 2 levels in ER-negative cells (19). Thus, lipocalin 2 may be more abundantly but not exclusively expressed in ER-positive cells.

Among the genes we identified lipocalin 2 appeared to be particularly interesting because of several features. Lipocalin 2 is a secreted protein and, therefore, may be a paracrine factor expressed by NER+ cells that affects the surrounding mammary epithelial cells. On the basis of prior studies lipocalin 2 may play a role in the regulation of cell proliferation and survival (20–23). Lipocalins are extracellular carriers of lipophilic molecules such as retinoids, steroids, and fatty acids, all of which are known to play important roles in the regulation of mammary cell growth (22, 24). To determine the effect of lipocalin 2 expression on mammary cell growth in vitro, we performed colony growth assays in MCF10A cells, a normal, immortalized ER− mammary epithelial cell line. We observed a 2–3-fold increase in colony numbers in cells constitutively expressing lipocalin 2 (data not shown), but the efficiency of obtaining stable colonies in MCF10A cells is very low making this result difficult to interpret. To determine whether exogenous treatment with lipocalin 2 would have similar effects to that of lipocalin 2 overexpression, we incubated MCF10A cells with conditioned medium obtained from GFP- or lipocalin 2-expressing cells. Similar to lipocalin 2 overexpression, exogenous addition of lipocalin 2 to MCF10A cells enhanced their...
growth (Fig. 2E) consistent with a paracrine mechanism of lipocalin 2 action. Although the ligand of lipocalin 2 and its mechanism of action are unknown, based on our colony growth experiments lipocalin 2 promotes the proliferation of ER− mammary epithelial cells.

To additionally analyze the relationship between lipocalin 2 expression and estrogen signaling, we analyzed the promoter region of the mouse and human lipocalin 2 genes, and identified a putative ERE with an almost perfect consensus ERE in both cases (Fig. 3A). To determine whether the putative ERE from the human gene can confer estrogen responsiveness to an exogenous gene, we placed the proximal lipocalin 2 promoter region containing or lacking this ERE up-stream of a luciferase gene (Fig. 3B). Measurement of luciferase activity after transient transfection of these constructs with cotransfection of ER in HepG2 human hepatoma cells revealed that the construct containing the ERE demonstrated modest ER responsiveness even in the absence of estrogen, which was abolished by the deletion of this sequence or the addition of estrogen antagonists ICI 128,780 and tamoxifen (Fig. 3C). Although the observed induction was modest, the differences seen in the fold induction between the Lipo-wERE-luc and Lipo-delERE-luc constructs, and between the untreated or estrogen-treated and the antiestrogen-treated groups were statistically significant (P < 0.001 for both cases based on Wilcoxon rank sum test). Similar results were obtained in T47D, MCF-7, and BT474 ER+ breast cancer cell lines (data not shown).

In summary, we isolated and characterized estrogen-responsive genes in normal human mammary epithelium and identified lipocalin 2 as a candidate paracrine factor that may mediate estrogen-induced proliferation in the normal mammary epithelium. Additional studies are required to determine the role of lipocalin 2 in estrogen signaling and the biochemical mechanism by which it influences cell growth. However, because lipocalin 2 is a secreted protein, it represents an attractive target for breast cancer prevention and treatment.

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References


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